Review

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Mitochondrial DNA heteroplasmy in disease and targeted nuclease-based therapeutic approaches

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Abstract

Mitochondrial DNA (mtDNA) encodes a subset of the genes which are responsible for oxidative phosphorylation. Pathogenic mutations in the human mtDNA are often heteroplasmic, where wild-type mtDNA species co-exist with the pathogenic mtDNA and a bioenergetic defect is only seen when the pathogenic mtDNA percentage surpasses a threshold for biochemical manifestations. mtDNA segregation during germline development can explain some of the extreme variation in heteroplasmy from one generation to the next. Patients with high heteroplasmy for deleterious mtDNA species will likely suffer from bona-fide mitochondrial diseases, which currently have no cure. Shifting mtDNA heteroplasmy toward the wild-type mtDNA species could provide a therapeutic option to patients. Mitochondrially targeted engineered nucleases, such as mitoTALENs and mitoZFNs, have been used *in vitro* in human cells harboring pathogenic patient-derived mtDNA mutations and more recently *in vivo* in a mouse model of a pathogenic mtDNA point mutation. These gene therapy tools for shifting mtDNA heteroplasmy can also be used in conjunction with other therapies aimed at eliminating and/or preventing the transfer of pathogenic mtDNA from mother to child.

Keywords gene editing; heteroplasmy; mitochondrial DNA
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See the Glossary for abbreviations used in this article.

Mitochondrial DNA

Human mitochondrial DNA (mtDNA), which was first discovered in 1963, is a 16,569-bp circular, double-stranded, supercoiled molecule which encodes for 37 genes, essential for oxidative phosphorylation (OXPHOS) and mitochondrial protein synthesis [1,2]. Of these 37 genes, 13 encode for subunits of 4 of the 5 multi-subunit enzymatic OXPHOS complexes located on the inner mitochondrial membrane: 7 subunits from Complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6); 1 subunit from Complex III (cytochrome b); 3 subunits from Complex IV (COXI, COXII, and COXIII); and 2 subunits from Complex V (ATP6 and ATP8). All subunits of Complex II are encoded by the nuclear genome (nDNA), along with all of the other subunits and assembly factors needed to complete the OXPHOS system [3]. In addition to these 13 subunits, the mtDNA also encodes for 22 tRNAs and 2 rRNAs. The mtDNA strands are referred to as the heavy strand (H-strand) and light strand (L-strand). The H-strand is guanine-rich, and the L-strand is cytosine-rich. [4]. There are 28 genes encoded by the H-strand and 9 genes encoded on the L-strand. Because of the dual origin of mitochondrial proteins, there must be a tight coordination between the two genomes for mitochondrial function; mutations in any of the protein-coding genes in either genome can lead to mitochondrial diseases, a heterogeneous group of diseases that are detailed below.

There is a high density of genetic information contained in the mtDNA due to the relatively small genome size, the number of encoded genes, and the lack of intron sequences. To accommodate this density, some of the genes lack complete termination codons or overlap one another [5]. Other features of the human mtDNA molecule include a non-coding regulatory region, referred to as the displacement loop (D-loop), two origins of replication (origin of replication of the H-strand (O_H), and origin of replication of the L-strand (O_L)), and two promoter sequences (heavy-strand promoter (HSP) and light-strand promoter (LSP)) [3]. The O_H, HSP, and LSP are all located within the D-loop region.

In most cells, there are approximately 1,000 mitochondrial genomes [6]. In contrast to the nDNA, these mtDNA genomes are not organized into chromosomes using histones; instead, the mitochondrial genome is organized in a DNA–protein structure called a nucleoid [7]. The majority of nucleoids contain only 1 mtDNA (on average each nucleoid has 1.4 mtDNA molecules). These mtDNA molecules are organized around a core set of proteins including TFAM (mitochondrial transcription factor A), POLG (mitochondrial DNA polymerase gamma), mtSSB (mitochondrial single-stranded binding protein), POLRMT (mitochondrial RNA polymerase), and LONP1 (mitochondrial Lon protease homolog) [8]. Another protein that is frequently found associated with the nucleoid is ATAD3, which directly or indirectly appears to mediate the association of the mtDNA nucleoid to the inner mitochondrial membrane [9,10]. The levels of mtDNA molecules are generally dependent on the cellular energy demands of a cell. mtDNA replication is independent of the cell cycle, and there are a few enzymes that are known participants in this process, including POLG and the mtDNA helicase

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Glossary

AAV9	adeno-associated virus
ATAD3	ATPase family AAA domain-containing protein 3
D-loop	displacement loop
DSB	double-strand break
HSP	heavy-strand promoter
H-strand	heavy strand
iPSC	induced pluripotent stem cell
KSS	Kearns–Sayre syndrome
LHON	Leber's hereditary optic neuropathy
LONP1	Lon protease homolog
LS	Leigh syndrome
LSP	light-strand promoter
L-strand	light strand
MELAS	mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF	myoclonus epilepsy, with ragged red fibers
MGME1	mitochondrial genome maintenance exonuclease 1
mitoRE	mitochondrial-targeted restriction endonuclease
mitoTALEN	mitochondrial-targeted transcription activator-like effector nuclease
mitoZFN	mitochondrial-targeted zinc-finger nuclease
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence
mtSSB	mitochondrial single-strand binding protein
NARP	neuropathy, ataxia, and retinitis pigmentosa
nDNA	nuclear DNA
O_H	heavy-strand origin of replication
O_L	light-strand origin of replication
OXPHOS	oxidative phosphorylation
PEO	progressive external ophthalmoplegia
PGC	primordial germ cell
POLG	polymerase gamma
POLRMT	mitochondrial RNA polymerase
PS	Pearson's syndrome
ROS	reactive oxygen species
RVDs	repeat variable diresidue
TALEN	transcription activator-like effector nuclease
TA	tibialis anterior
TFAM	mitochondrial transcription factor A
ZFN	zinc-finger nuclease
ΔmtDNA	deleted mitochondrial DNA

TWINKLE [11–14]. Despite the knowledge of enzymes that play a role, the mechanisms regulating mtDNA copy number have remained elusive.

Compared to the nuclear genome where mendelian inheritance is the norm, the pattern of mitochondrial genome inheritance is uniparental, specifically maternally inherited. The unit of inheritance is the mtDNA nucleoid [7]. Following fertilization, there is a loss of sperm mitochondria, thereby blocking paternal mtDNA transmission. This loss can be active, through degradation of the sperm mitochondria through ubiquitination, mitophagy, or mtDNA degradation, or passive, through the dilution of paternal mtDNA into the maternal mtDNA pool [15,16]. Historically, there has been one report of paternal mtDNA transmission in humans, suggesting that this degradation and subsequent blockage can be overcome; however, this is not a common occurrence [17]. More recently, three unrelated multigenerational families have been identified with biparental mtDNA transmission, that is similar to an autosomal dominant-like inheritance pattern [18]. This interpretation has been challenged by the identification of large nuclear pseudogenes (a consequence of evolutionary migrations of mtDNA fragments to the nucleus) that could explain the inheritance of paternal mtDNA sequences [19–22].

Mitochondrial DNA heteroplasmy

Compared to the nDNA, mtDNA has a high sequence evolution rate (approximately 10–20 times higher), which can partly be attributed to the 100- to 1,000-fold higher mutation rate in the mtDNA [23–25]. These mutated mtDNA molecules tend to form sporadically, and due to the high copy number, mutated and wild-type mtDNA molecules can exist together in a single cell (mtDNA heteroplasmy) [26]. Because mtDNA replication is cell-cycle-independent and mtDNA can be segregated during replication, heteroplasmy levels are dynamic and can change during a lifetime in both mitotic and post-mitotic cells/tissues [11]. Along with the nature of the mutation, the percentage of heteroplasmy is the major factor which determines the clinical severity of mitochondrial diseases. There is a biochemical threshold associated with mutant mtDNA percentage that must be surpassed for decreased OXPHOS function and phenotype onset [27]. This threshold is dependent on the mutation, the cell type, and tissue type, and can vary between 60 and 90% mutant mtDNA for an detectable phenotype to present [28]. Different tissues have different biochemical thresholds, and therefore, a bioenergetic defect may be seen in certain tissues before others in a patient. Additionally, the percent heteroplasmy can shift during mitotic and meiotic cell division, leading to an array of bioenergetic defects depending on a cell's or tissue's replicative capacity.

Mitochondrial DNA segregation alters heteroplasmy in the germline

Understanding mitochondrial dysfunction can be an arduous task because of the complex interactions between the nuclear and mitochondrial genomes. The fact that mtDNA can segregate both along the maternal germline and in somatic tissues adds further complexity. In understanding heteroplasmic variation in the maternal line, it is first important to recognize that individual oocytes can have varying heteroplasmy levels. In a first study, 82 oocytes from a mother harboring both the m.3243A>G (18.1% heteroplasmy in quadriceps and 7.2% in leukocytes) mutation (discussed in more detail below) were analyzed [29]. These oocytes had a range of 0–45% heteroplasmy for the m.3243A>G mutation, with a mean of 12.6% and median of 8.2% heteroplasmy, while 8 oocytes lacked any detectable mtDNA mutation levels [29]. The authors of this study modeled the heteroplasmy distribution across the oocytes as a binomial distribution assuming that the initial maternal mutant allele frequency was the mean of the oocytes analyzed. In a second study, a mother harboring the m.8993T>G (50% heteroplasmy in blood) mutation (discussed in more detail below) was hyper-ovulated and the resultant 7 oocytes were analyzed for heteroplasmy levels [30]. One oocyte was determined to not harbor the mutation, while the other 6 had > 95% heteroplasmy for the m.8993T>G point mutation [30]. Interestingly, the children from these mothers have their own variation in heteroplasmy, where the mother with the m.3243A>G mutation (from the first study) had a son with 11.7% of the m.3243A>G mutation in blood, while the mother with only the m.8993T>G mutation (from the second study) had three sons, one with 98% of the mutation in quadriceps and fibroblasts (died from Leigh syndrome (a mitochondrial disease discussed in detail

below)), one with 95% in blood (died of sudden infant death syndrome), and one with 87% in blood who was affected by Leigh syndrome [29,30]. This wide spectrum of shifts in heteroplasmy between generations raises the question how (and if) certain pathogenic mtDNA species segregate in oocytes?

Mitochondrial DNA germline bottleneck

This variability in heteroplasmy in children can be attributed in part to the mtDNA segregation following fertilization, as an embryo generated from an oocyte that is heteroplasmic for a pathogenic mtDNA species will have different levels of mtDNA heteroplasmy in the different developing tissues and organs depending on the mtDNA segregation following fertilization. Part of this is due to the mtDNA "bottleneck" that occurs once an oocyte has been fertilized. The genetic bottleneck theory is based on evidence from Holstein cows that only a significant reduction in mtDNA copy number could be responsible for the large shifts in mtDNA heteroplasmy over a few generations [31]. Further studies done on the genetic bottleneck in mice have been controversial, with different studies finding that the extent of mtDNA copy number reduction is variable. Some have shown that this reduction in copy number goes from 100,000 mtDNA copies in a fertilized oocyte to 200 in the primordial germ cell (PGC) (about 40 mitochondria with 5 mtDNA copies each), while others have demonstrated a copy number reduction, but only to 1,500 mtDNA molecules per cell [32–35]. It has been recently shown that these differences in the extent of copy number reduction during the germline bottleneck could be explained by the diverse nuclear genotypes of the inbred mouse strains used in these studies [36]. Due to ethical considerations, up until recently *in vivo* studies to determine the extent of the genetic bottleneck during germline development have not been carried out in humans. Methodologies to isolate PGCs from human embryos still have not been successful, but protocols to isolate human PGCs from Carnegie stage 12 and onwards have been developed and allow for the investigation of the human germline [37,38]. Using adjacent-section electron micrographs and super-resolution whole-cell imaging on PGCs isolated *in vivo* from healthy female embryos, the Chinnery group observed a profound reduction in mtDNA content, determined to be ~5 mtDNA molecules per mitochondria, which did not change between Carnegie stages 12 and 20–21 [37].

Further analysis of the mtDNA in these PGCs indicated that these healthy female embryos harbored low-level mtDNA heteroplasmy, most of the variants were nucleotide transitions. Additionally, in PGCs from Carnegie stages 12 and 20–21 there was a decrease in the proportion of non-synonymous variants in protein-coding genes and a decrease in the mutation rate per base for tRNA genes—both of which indicate selection against potentially harmful mtDNA variants. Surprising to the authors, there was evidence of selection against variants in the D-loop region of the mtDNA, which is non-coding. This selection process occurring early during development exposes potentially harmful mtDNA variants to selection, preventing the accumulation of mtDNA mutations; however, mutations that escape this selection process are likely the cause of the extreme heteroplasmy shifts that can be seen from one generation to the next. It was recently reported that heteroplasmy segregation can fall on a spectrum, ranging from random drift to direct selection, depending on the mitochondrial–nuclear interactions, with the goal of achieving maximal mitochondrial fitness [36]. An additional study using mouse models with two distinct mtDNA haplotypes showed an increase in both germline heteroplasmy variance in oocytes and mtDNA segregation between germline and a reference tissue as the age of the mother increased [39].

While it is known that the genetic bottleneck occurs during development, the mechanism(s) remains elusive. Three potential mechanisms, that are not mutually exclusive, have been proposed: (i) the passive reduction of mtDNA; (ii) the focal replication of mtDNA; and (iii) packaging of mtDNA into larger segregating units (reviewed in Ref. [40]). All of these mechanisms (together or separately) could lead to large changes in heteroplasmy within one generation (Fig 1).

Mitochondrial selection alters heteroplasmy in the germline

In addition to mitochondrial segregation in the germline, selection at the mitochondrial level also can be responsible for heteroplasmy variations. Using whole-genome sequencing of nearly 13,000 individuals, it was determined that depending on the location of the heteroplasmic variant, the likelihood of transmission was altered [22]. Heteroplasmic variants in the D-loop region were more likely to be transmitted than variants found in the rRNA genes, while transmission of variants in protein-coding genes and tRNAs fell somewhere in between. When analyzing protein-coding genes, if the variant was a synonymous variant, it was more likely to be transmitted compared with non-synonymous variants. In the > 1,500 mother–offspring pairs that were analyzed in this study, the heteroplasmic variants were defined to fall into 1 of 3 categories: (i) transmitted/inherited (if the variant was found in both the mother and the offspring); (ii) lost (if the variant was present only in the mother); or (iii) *de novo* (if the variant was only found in the offspring). Analysis of these variants showed that both, in the mother and the offspring, the fraction of lost or *de novo* variants were significantly lower than the transmitted or inherited variants. Interestingly, of the heteroplasmic variants that were analyzed, those that had previously been described (in the 1,000 Genomes datasets and Single Nucleotide Polymorphism Database) were more likely to be transmitted/inherited than those that were previously not observed.

Mitochondrial DNA-based diseases

Mitochondrial DNA point mutations and their associated diseases

mtDNA point mutations can arise from inefficiency of the mtDNA repair system, replication errors, or reactive oxygen species (ROS)-induced oxidative damage [41–43]. Point mutations can either be maternally inherited or occur sporadically. Most pathogenic mtDNA point mutations exist in heteroplasmy. The prevalence of mtDNA point mutations ranges between 1:5,000 and 1:500,000 [26,44]. Over the last 30 years, over 330 pathogenic point mutations have been identified in the human mtDNA, and this number is ever-increasing [3].

The first report of an inheritable mtDNA-based disorder was Leber's hereditary optic neuropathy (LHON) [45]. Across the mtDNA, there are many point mutations associated with LHON, but three of the most common, which tend to be present as homoplasmic mutations, are m.11778G>A (ND4), m.3460G>A (ND1), and m.14484T>C (ND6) [46,47]. Point mutations which tend to be heteroplasmic that are attributed to LHON are m.14459G>A and

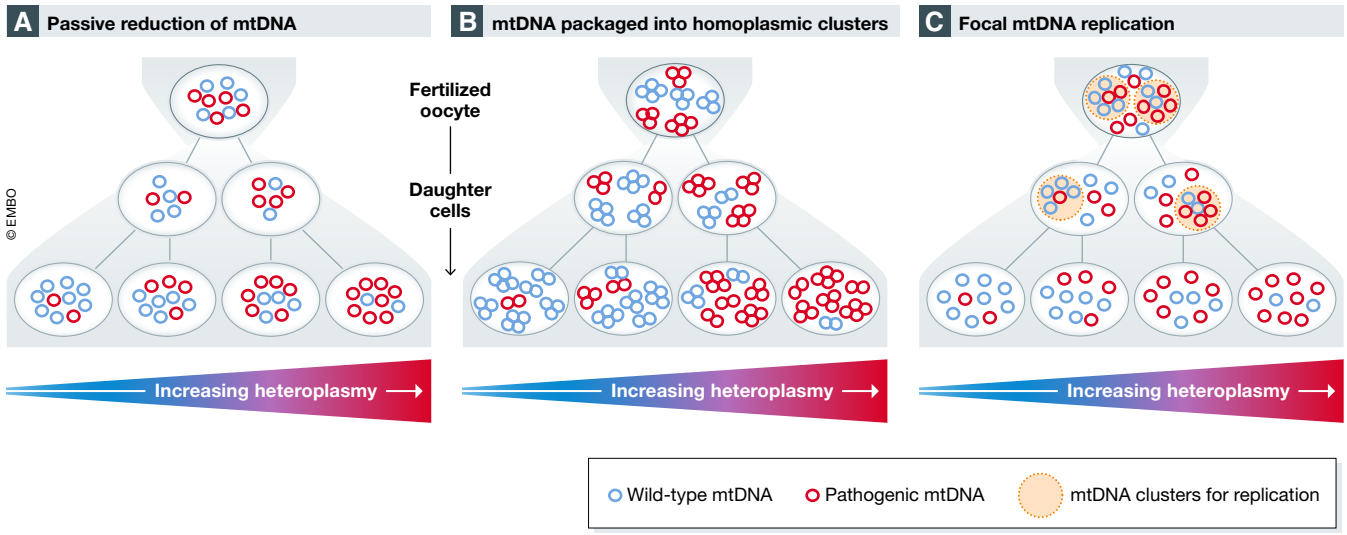

Figure 1. Mechanisms of mtDNA depletion during genetic bottleneck resulting in daughter cells with varying heteroplasmy.

During the genetic bottleneck that occurs during germline development, there is a reduction in mtDNA levels in the fertilized oocyte and the resulting daughter cells have a wide range of heteroplasmy levels. Some possible mechanisms of the reduction in mtDNA that can explain the bottleneck during germline development include (A) the passive but marked reduction in mtDNA levels during each cell division in early development, followed by stochastic segregation of mtDNA into daughter cells; (B) mtDNA packaged into homoplasmic clusters which are reduced in discrete segregating units during each cell division; and (C) focal mtDNA replication where only a selected population of mtDNA molecules are replicated. All of these possible mechanisms would result in daughter cells with varying heteroplasmy.

m.14600G>A (both in ND6). For the mutations that are found in heteroplasmy, a high mutation load (> 80%) must be observed for a clinical phenotype to be seen [48]. LHON is considered to be one of the most prevalent of the mitochondrial diseases. It is primarily seen in young males, and patients exhibit acute or subacute bilateral vision loss [47].

Leigh syndrome (LS) is a unique clinical presentation, but it can be attributed to mutations in the mtDNA or the nDNA [49]. Many of the mtDNA point mutations associated with LS are in Complex I genes, including m.3697G>A (ND1), m.10191T>C (ND3), and m.13513G>A (ND5); other associated mtDNA point mutations are m.9176T>C and m.8993T>G/C (both in ATP6) [50–53]. For a pathogenic phenotype to present, heteroplasmy levels must be > 80% for these point mutations [27]. Primarily, patients with LS present with subacute neuronal degeneration of the basal ganglia leading to hypotonia, spasticity, movement disorders, cerebellar ataxia, and peripheral neuropathy [49,54].

Neuropathy, ataxia, and retinitis pigmentosa (NARP) is most often associated with the m.8993T>G/C (ATP6) point mutation that is also associated with LS when present at high levels (> 80%) [55]. Patients with NARP exhibit symptoms of proximal neurogenic muscle weakness with sensory neuropathy, ataxia, and pigmentary retinopathy [49].

Eighty percent of mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes (MELASs) patients have the m.3243A>G ($tRNA^{Leu(UUR)}$) [56]. Heteroplasmy levels exceeding 60% can lead to a pathogenic phenotype [27]. MELAS patients exhibit a progressive encephalopathy and stroke-like episodes [57–59]. Besides MELAS, the same mutation has been shown to also cause a wide spectrum of clinical manifestations, from isolated myopathy to isolated diabetes, hearing loss, migraines, or multisystem encephalopathies [57,60,61].

Another mitochondrial disorder characterized by a point mutation in a tRNA gene is myoclonus epilepsy, with ragged red fibers (MERRFs). MERRF is most commonly associated with the m.8344G>A ($tRNA^{Lys}$) mtDNA mutation, and >70% heteroplasmy for this mutation can lead to pathogenesis [27,62,63]. MERRF patients exhibit myoclonus epilepsy as well as generalized seizures, hearing loss, eyelid ptosis, and multiple lipomatosis [64,65].

Mitochondrial DNA deletions and their associated diseases

Deletions in the mtDNA are damaging because they remove many protein-coding genes as well as tRNA and rRNA genes. These deletions can vary in size from 1.8 to 8 kb, and can exist anywhere in the mtDNA, though there are some areas of the mtDNA that are more susceptible to deletion formation and origins of replication need to be conserved [66–68]. The molecular mechanisms underlying the formation of deleted mtDNA (ΔmtDNA) are under active investigation. Many mtDNA deletion breakpoints are flanked by direct or imperfect repeats in the intact mtDNA (Class I deletions), implying that mtDNA deletions can form from replication slippage [15,69,70]. In 2017, single-molecule analysis of mtDNA showed replication stalling at the junction of a mtDNA deletion, suggesting impaired replication could be the mechanistic basis of Class I mtDNA deletion formation [70]. More recently, it has been demonstrated that mispairing during L-strand synthesis during mtDNA replication is likely responsible for deletions involving direct repeats [71]. On the other hand, mtDNA deletions not involving direct repeats (Class II deletions) appear to be formed by double-strand breaks and re-ligation of free DNA ends. This has been clearly shown in mouse models expressing mitochondrial-targeted restriction endonucleases [72,73].

The formation of deletions is usually sporadic, and these are generally not thought to be maternally transmitted [74]. However, from a

study done in 2004 analyzing 226 families where a single species of mtDNA deletion had been identified, it can be inferred that the actual risk of transmission to the offspring is approximately 1 in 24 births [75]. These large mtDNA deletions or rearrangements are thought to exist in heteroplasmy [76,77]. If heteroplasmy is particularly high for a mtDNA deletion in a young patient, the prevailing theory is that the deletion occurred early in development, shortly after the bottleneck and expanded to high heteroplasmy with the potential to affect many tissues [76]. Accumulation of mtDNA deletions in post-mitotic tissues is also observed during normal aging [78–82].

The most prevalent deletion found in patients is the common deletion. The common deletion is a 4,977-bp deletion (Δ4,977) whose breakpoint is flanked by 13-bp direct repeats [67,74,83]. The common deletion is also found at low levels in post-mitotic tissues of most aged individuals but at lower levels than in bona-fide mitochondrial diseases. It results in the loss of 12 essential mitochondrial genes, including OXPHOS genes (ATP8, ATP6, COXIII, ND3, ND4L, ND4, and ND5) and tRNAs (G, R, H, S2, and L2). The three most associated syndromes with the common deletion are Pearson's syndrome (PS), Kearns–Sayre syndrome (KSS), and progressive external ophthalmoplegia (PEO). PS is characterized by sideroblastic anemia with vacuolization of bone marrow precursor cells and pancreatic dysfunction with early onset [84,85]. PS is usually fatal in infancy, but patients who survive through childhood often develop KSS. KSS is a multisystemic disorder with onset before 20 years of age. Patients with KSS suffer from an oculocraniosomatic disorder characterized by myopathy, pigmentary retinopathy, and cerebellar ataxia and cardiac conduction defects [66]. PEO is a myopathy which impacts a patient's ability to move their eyes and eyelids [86]. There is no particular age of onset, but PEO can develop over the course of 5–15 years and these patients usually have other signs of generalized myopathy.

Mitochondria-targeted nuclease-based therapeutic interventions for mitochondrial disease

Currently, there are no cures for mitochondrial diseases, only palliative treatments which can lessen the effects of these debilitating disorders. As discussed above, mitochondrial diseases can arise from mutations or deletions in either the mtDNA or the nDNA and with the advent of gene therapy tools, and efforts have been made to fix or replace mutated genes to treat patients. The heteroplasmic nature of most mtDNA-based disorders creates a unique opportunity for therapy, which, instead of trying to fix the damaged gene, targets the entire mutant molecule for degradation and allows the wild-type molecules to repopulate and restore the copy number. Over the last 20 years, the use of double-strand breaks (DSBs) to shift mtDNA heteroplasmy has been validated in a number of different models. Mitochondrially targeted restriction endonucleases (mitoREs) have been used to generate DSBs to cleave at specific mtDNA sequences to quickly deplete a single species of mtDNA in *in vitro*, *ex vivo*, and *in vivo* mouse models of mtDNA heteroplasmy [87–91]. The m.8993T>G mutation associated with NARP generates a *Sma*I/*Xma*I site that is not found in the wild type. When mito-*Sma*I/mito*Xma*I was delivered to cybrids (cytoplasmic hybrids) harboring > 90% of the mutation, there was a reduction in the mutant molecules and a corresponding increase in the wild-type mtDNA molecules [92,93]. While effective at shifting mtDNA heteroplasmy, this method depends on the presence of a naturally occurring unique restriction site that encompasses the point mutation or deletion, which is not very common.

Key to changing mtDNA heteroplasmy with specific nucleases is the fact that mitochondria do not have a DSB repair system. In fact, following DSB, mtDNA is rapidly degraded [87]. Recently, it was found that this degradation is mediated by the enzymes associated with mtDNA replication, including the exonuclease activity of polymerase gamma and the nuclease Mgme1 [72,94]. The mtDNA copy number control is poorly understood, but upon mtDNA depletion, the residual molecules show increase replication in order to normalize the copy number [72,90,95–99]. Because residual molecules are predominantly wild type after the destruction of mutant mtDNA, this recovery in copy number leads to an increase in wild-type mtDNA ratios and improved mitochondrial function (Fig 2A).

Dimeric nucleases for gene therapy interventions

Recently, focus has been given to designer nucleases, such as transcription activator-like effector nucleases (TALENs) or zinc-finger nucleases (ZFNs) which can be targeted to the mitochondria and engineered to bind to specific but longer DNA sequences, giving them an advantage over mitoREs [95–97,99–101]. Our laboratory has developed mitochondria-targeted TALEN (mitoTALEN) to target a mtDNA point mutation associated with LHON and dystonia (m.14459G>A) and the mtDNA "common deletion" (m.8483-13459del4977) in patient-derived cybrids [96]. Subsequent studies in patient-derived cybrids have been carried out using mitoTALEN to target other point mutations including m.8344A>G and m.13513G>A which are associated with MERRF and MELAS/LS, respectively [100].

mitoTALENs are comprised of a sequence-specific modular DNA-binding domain, and they contain a sequence-independent endonuclease domain from *Fok*I (Fig 2B). *Fok*I has been engineered to act as an obligate heterodimer so two mitoTALEN monomers must be in close proximity for *Fok*I to dimerize and create DSBs in the mtDNA [102]. The TALE sequence-specific DNA-binding domains of TALENs are repeats of modular DNA-binding domains (called repeat variable diresidue, or RVDs) which can be engineered to recognize a specific nucleotide [103,104]. While mitochondria-targeted ZFN (mtZFN or mitoZFN) architecture is very similar to mitoTALEN, the two main differences are as follows: (i) Each DNA recognizing domain can recognize 3 nucleotides and (ii) the ZFNs require a nuclear export signal in addition to the mitochondrial targeting sequence (MTS) to target the protein out of the nucleus and into the mitochondria [105]. Also commonly included in the mitoTALEN of mitoZFN architectures is an immunological tag at the N-terminus of each monomer (either HA or FLAG), after the MTS and an independent fluorescent marker (either eGFP or mCherry) to select for expression. Transiently transfected cells which express both eGFP and mCherry are selected by cell sorting, and changes in heteroplasmy and mtDNA levels can be assessed. Figure 2B summarizes the overall structure of the different DNA editing platforms used for the elimination of mutant mtDNA.

In the case of a mtDNA point mutation, one DNA-binding domain (or monomer) of mitoTALENs or mitoZFNs will recognize the mtDNA sequence which harbors the point mutation, and the other will recognize a wild-type mtDNA sequence that is in close proximity for the *Fok*I nucleases to dimerize and cleave the

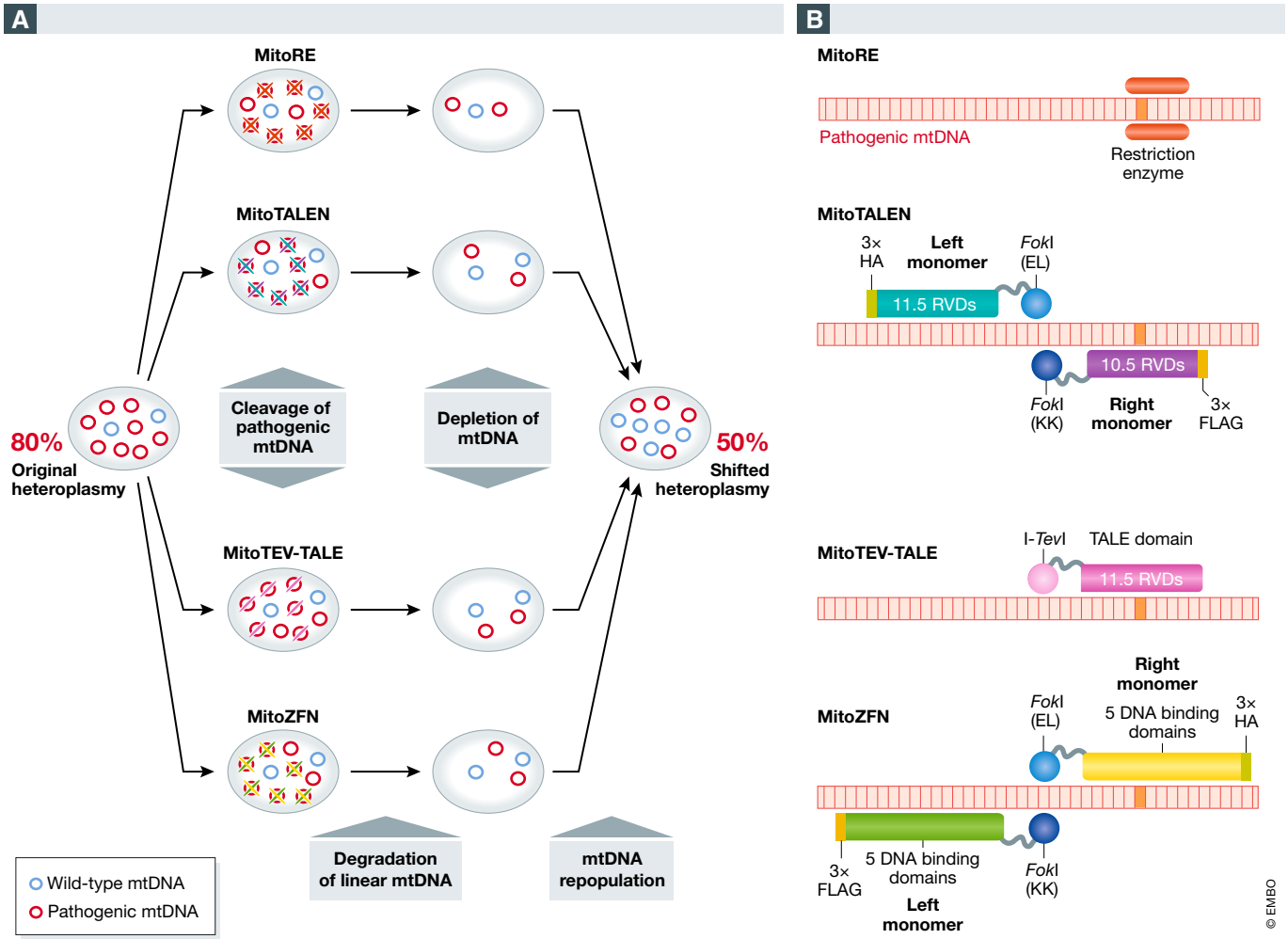

Figure 2. Mechanism of mtDNA heteroplasmy shift following DNA editing enzyme-mediated DSB.

Targeting pathogenic mtDNA for degradation is one method of shifting mtDNA heteroplasmy below the biochemical threshold. (A) Staring with a cell with high levels of heteroplasmy, mitoREs, mitoTALENs, mitoTEV-TALEs, and mitoZFNs can be used to selectively or preferentially cleave the pathogenic mtDNA molecules, resulting in a transient depletion of total mtDNA levels. Mechanisms associated with copy number control will return mtDNA to pre-therapeutic levels but with a lower load of the pathogenic mtDNA. (B) Architecture of the DNA recognition elements and DNA editing enzymes used to shift mtDNA heteroplasmy. mitoREs are enzymes that bind to and cleave mtDNA at a specific, but short recognition sequence. mitoTALENs are comprised of two monomers, each with a DNA recognition element and one *Fok*I monomer, when two *Fok*I monomers are sufficiently close together they will dimerize and cleave the mtDNA. mitoTEV-TALEs are comprised of a single monomer, with a DNA recognition element and the I-*Tev*I nuclease which can cleave mtDNA at a CNNNG sequence. mitoZFNs are similar to mitoTALENs in architecture, where there are two DNA recognition elements and two *Fok*I nucleases that need to dimerize to cleave the mtDNA sequence. The difference between the DNA recognition elements in mitoTALENs/mitoTEV-TALEs and mitoZFNs is the number of nucleotides recognized by each element (1 versus 3 nucleotides).

mutated mtDNA molecule. In the wild-type mtDNA, only one of the monomers (the "wild-type" monomer) would bind to the mtDNA, and since there is no second *Fok*I domain to constitute a functional dimer, a DSB should not occur. In the case of a mtDNA deletion, each monomer would recognize a DNA sequence that is flanking either side of the deletion breakpoint, in close enough proximity to one another for the *Fok*I-mediated DSB to cleave the mtDNA. In the wild-type mtDNA molecule, these monomers will bind far enough away from one another that there should be no mtDNA cleavage.

mitoTALENs and mitoZFNs have been used to shift heteroplasmy in a number of different patient-derived pathogenic mtDNA models (Table 1). In the m.14459G>A model, cells that were transiently transfected with the specific mitoTALEN monomers and sorted for both eGFP and mCherry expression, showed not only a significant shift in heteroplasmy toward the wild-type mtDNA, but also any mtDNA depletion that was seen 48 h after transfection was reversed to non-transfected status by 14 days after transfection [96]. When using a mitoTALEN that was not specific to the m.14459G>A point mutation, there was no mtDNA depletion, nor shift in heteroplasmy. In the m.8993T>G model, cells that were transiently transfected with specific mitoZFN monomers and sorted for GFP and mCherry expression also showed a significant shift toward the wild-type mtDNA and the depletion seen at 24 h returned to non-transfected levels by 28 days after transfection [98]. Further, both our group and the Minczuk group were able to use mitoTALEN and mitoZFN

Table 1. Engineered mito-nucleases used in human mtDNA mutation/deletion models.

	Model tested	Delivery	*In vitro*	*In vivo*	Results	Citation
mitoRE						
m.8993T>G NARP mutation	Osteosarcoma cybrids, > 90% mutation	Transfection/infection	Yes	No	Reduction mutant haplotype.	[92,93]
BALB/NZB mouse	Several	Transfection/infection	Yes	Yes	Reduction in target haplotype	[87–91]
mitoTALEN						
"Common deletion" (m.8483_13459del4977) breakpoint (Δ5)	Osteosarcoma cybrids, 70–80% mutation	Transfection	Yes	No	Reduction mutant haplotype	[96]
m.14459G>A *ND6* mutation	Osteosarcoma cybrids, 90–95% mutation	Transfection	Yes	No	Reduction mutant haplotype. Complex I activity recovery	[96]
m.8344A>G $tRNA^{Lys}$ mutation	Osteosarcoma cybrids, 55–60% mutation	Transfection	Yes	No	Reduction mutant haplotype OXPHOS recovery	[100]
m.13513G>A ND5/MELAS mutation	Osteosarcoma cybrids, 80–85% mutation	Transfection	Yes	No	Reduction mutant haplotype. Complex I activity recovery	[100]
m.14459G>A LHOND mutation	Fusion mouse oocytes, 80–85% human mutation	Injection of RNA in oocytes	Yes	Yes	Reduction mutant haplotype	[91]
m.9176T>C NARP mutation	Fusion mouse oocytes, > 95% human mutation	Injection of RNA in oocytes	Yes	Yes	Reduction mutant haplotype	[91]
m.3243A>G $tRNA^{Leu(UUR)}$/MELAS mutation	Human IPSc, > 80% mutation	Transfection	Yes	No	Reduction mutant haplotype. OXPHOS recovery	[106]
m.13513G>A *ND5*/MELAS mutation	Human IPSc, 60–70% mutation	Transfection	Yes	No	Reduction mutant haplotype	[107]
mitoZFN						
"Common deletion" (m.8483_13459del4977) breakpoint (Δ5)	Osteosarcoma cybrids, 70–80% mutation	Transfection	Yes	No	Reduction mutant haplotype	[98]
m.8993T>G NARP mutation	Osteosarcoma cybrids, > 90% mutation	Transfection	Yes	No	Reduction in mutant haplotype. OXPHOS recovery	[98,99]
mito*Tev*-TALE						
m.8344A>G $tRNA^{Lys}$ mutation	Osteosarcoma cybrids 90% mutation	Transfection	Yes	No	Reduction mutant haplotype OXPHOS recovery.	[111]

to effectively shift mtDNA heteroplasmy in cell models of the common deletion [96,99]. mitoTALENs have also been used in two different iPSC models of mutations associated with MELAS, where transfection of mitoTALENs resulted in the reduction in the specific mutant haplotype [106,107].

More recently, both mitoTALEN and mitoZFN have been taken forward into an *in vivo* model of a mtDNA point mutation, the m.5024C>T mouse model described previously [95,97,108]. This mouse model develops a mild cardiomyopathy with age, but the most striking biochemical phenotypes are the reduction in the steady-state levels of the mitochondrial $tRNA^{Ala}$ and decreased *in organello* protein synthesis. Both groups used recombinant adeno-associated virus 9 (referred to as AAV9)-mediated viral delivery of each monomer of their respective DNA editing tool against the mutated mtDNA molecule. The AAV9 serotype used has high tropism for skeletal muscle [109,110]. Intramuscular injections of mitoTALEN into the tibialis anterior (TA) showed a significant shift in mtDNA heteroplasmy toward the wild type at 10, 12, and 24 weeks after injection compared with either AAV9-*GFP* or single AAV9-mitoTALEN monomer-injected TA [95]. This shift in heteroplasmy was accompanied by a corresponding increase in the mitochondrial $tRNA^{Ala}$ levels. More importantly though, systemic injections of mitoTALEN done through the retro-orbital sinus (systemic venous system delivery) also showed a significant shift in heteroplasmy in the heart at 6 and 12 weeks post-injection, and in the quadriceps at 12 weeks post-injection. Tail vein injections of the mitoZFNs showed a significant shift in mtDNA heteroplasmy in the heart toward the wild type 65 days after injection. Interestingly, with increasing amounts of AAV9 this shift was accompanied by a depletion of mtDNA copy number in the heart, suggesting that there is a dose dependence to these gene editing enzymes and at higher titers there may be non-specific cleavage of the wild-type mtDNA (in addition to cleaving the mutated mtDNA molecules) [97].

Monomeric nucleases for gene therapy interventions

The successful systemic delivery of mitoTALEN and mitoZFN *in vivo* to shift mtDNA heteroplasmy and restore tRNA levels is a major hurdle to pass in order to move toward clinical use of these gene therapy tools in patients. Another obstacle which can be minimized to make mtDNA gene therapy more efficient is to find ways to deliver nucleases which do not need to dimerize in order to cleave the mtDNA, reducing the complexity of the recombinant viruses that need to be delivered. Our laboratory has published on the use of the I-*Tev*I nuclease to shift mtDNA heteroplasmy [111]. In

the mito*Tev*-TALE construct, the TALE domain is linked to the I-*Tev*I nuclease instead of the *Fok*I endonuclease. I-*Tev*I acts as a monomer and creates a DSB at a CNNNG site in the mtDNA. A mito*Tev*-TALE targeting the MERRF m.8344A>G mutation could significantly shift heteroplasmy toward the wild type and restored mitochondrial function in a cybrid cell model [111] (Fig 2B).

Efforts using technologies such as CRISPR have not yet been successful because of difficulties targeting the guide RNA into the mitochondria [112], although a single report claims to have it working [113].

Minimizing off-target effects when using mitochondrial gene therapy interventions

In translating these gene therapy tools from *in vitro* and *in vivo* models of mtDNA heteroplasmy into patients, care has to be taken to ensure there are no off-target effects of the DSBs either in the mitochondrial genome or in the nuclear genome. As mentioned above, after generation of double-strand breaks in the mtDNA, there is a transient mtDNA depletion. This depletion has been observed using mitoREs, mitoTALENs, and mitoZFNs, but after a sufficient recovery time (days to weeks) this depletion is no longer observed [90,95–100,111]. Although most mtDNA is degraded after double-strand breaks, rare mtDNA fragments can form deleted species, which have been observed at very low levels [73,114]. The presence of these low levels of deletion would not negate the major benefit to mitochondrial function triggered by mutant mtDNA elimination in a heteroplasmic environment.

Minimizing any off-target effects of mitoTALENs or mitoZFNs in the nDNA is more important since there are only two copies of each gene in a cell compared with the mtDNA. Even though there are more DNA repair mechanisms in place in the nucleus, a double-strand break in the nDNA would nevertheless pose a larger problem for patients. The best way to prevent this from occurring is to employ *in silico* analysis of similar sequences in the nDNA (mtDNA pseudogenes) and choosing appropriate targeting sequences that are specific to the mtDNA. Gammage *et al* found that two potential nuclear targets were not affected after mitoZFN treatment *in vivo* [97].

Considerations for gene therapy to supplement other mitochondrial therapeutics

In addition to the advances in mitochondrial gene therapy to treat mitochondrial diseases, mitochondrial replacement therapies and techniques are being explored—one of which has gotten a lot of attention lately is the mitochondrial replacement in oocytes/embryos. If a woman has high levels of pathogenic mtDNA, a donor egg with healthy mtDNA can be enucleated and the nuclear genome replaced with that of the woman with the pathogenic mtDNA before being fertilized. In theory, this technique should eliminate the transmission of the pathogenic mtDNA to the next generation, as only < 2% of the pathogenic mtDNA remains after the transfer (from the small amount of cytoplasm surrounding the nucleus in the karyoplast used for transfer) [115,116]. A caveat to this mitochondrial replacement therapy is ensuring that the donor mtDNA is compatible with the nuclear genome of the recipient. Discrepancies in the mitochondrial–nuclear genomes can result in decreased mitochondrial bioenergetics, elicit an immune response, or affect other processes, such as aging [117,118]. Spindle transfer resulted in > 99% donor mtDNA in oocytes, and in most cases, this donor mtDNA was stably maintained in embryonic stem cells generated from these oocytes [119]. However, around 15% of these ES cells demonstrated a gradual loss of the donor mtDNA and a complete reversal toward the pathogenic maternal haplotype, a phenomenon seen by several groups [115,116,120]. To prevent this reversion from occurring in embryos after fertilization, mitochondrial gene therapy tools can be used following spindle or pronuclear transfer to completely eliminate the pathogenic mtDNA species. Both mitoRE and mitoTALEN have been used in embryos and were found to eliminate specific mtDNA haplotypes [91].

Box 1. In need of answers

- Since different tissues can have different biochemical thresholds for pathogenic mtDNA, can these different tissues also differentially regulate mtDNA heteroplasmy levels?
- Which enzymes participate in the formation of mtDNA deletions after DSB?
- How to move mitochondrial-targeted nuclease-based therapeutics to the clinic?
- During mitochondrial replacement therapies, how does the mitochondrial reversion occur after a nearly complete replacement of pathogenic mtDNA?

Concluding remarks

Mitochondrial diseases are a heterogeneous group of diseases that can manifest at any age, affect almost any tissue, and have varied clinical pathologies. In recent years, advances have been made to understand how mtDNA can segregate in the germline, during development, and in cells and tissues to affect the phenotypic outcome in a patient. In addition to gaining a better understanding of the basis of mitochondrial diseases, therapeutic approaches including gene therapies and mitochondrial replacement have been explored and expanded upon. Together, the understanding of both how mitochondrial diseases arise from pathogenic mtDNA variants and methods to safely and effectively remove/replace the pathogenic mtDNA will aid in the ongoing fight against mitochondrial diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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